Methods for the detection of botulinum neurotoxin activity: BoTest™, BoTest™ Matrix, and BoCell™ Assays

ICCVAM Submission
June 2011

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BoNT detection:

BioSentinel's scientific and business goals:

1. Develop assays for the detection of BoNT
   - activity-based focus
   - quantitative, when possible
   - minimal equipment requirements
   - near mouse bioassay sensitivity

2. Utilize assays for the development of post-exposure therapeutics, BoNT-based therapies, environmental testing methodologies, and biodefense detection systems.

3. Qualify and commercialize the assays
   a. BoTest™ and BoTest™ Matrix (*in vitro*)

   b. BoCell™ (cell based)

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Product overview:

1. BoTest™ (2 products: BoTest™ A/E and BoTest™ B/D/F/G)
   - Detects BoNT proteolytic activity
   - mix-and-read, no secondary reagents
   - Detection of six of seven serotypes
   - <100 fm - 10 pM sensitivities
   - Drug discovery, specific activity determinations, drug product quantification

2. BoTest™ Matrix (2 products: BoTest™ Matrix A and E)
   - Detects BoNT proteolytic activity with heavy-chain identification
   - Immunoprecipitation followed by BoTest™ detection
   - Detection of serotypes A and E (others in development)
   - <100 fm - 1 pM sensitivities
   - Detection and quantification of BoNT in complex matrices

3. BoCell™ A Cell-based Assay
   - Detects BoNT proteolytic activity after cell receptor binding, internalization, and translocation.
   - mix-and-read, no secondary reagents
   - Detection of serotype A (BoNT/B assay in development)
   - 30 - 300 pM sensitivity
   - Drug discovery, specific activity determinations, drug product quantification

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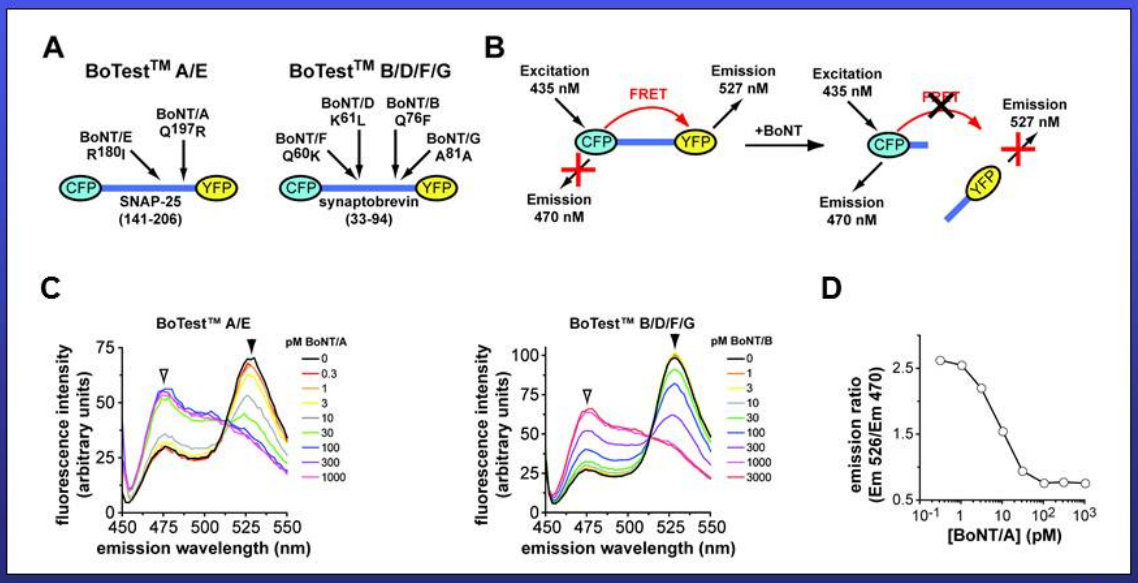
BoTest™ BoNT Detection Reporters: *in vitro* detection of serotypes A, B, D, E, F, and G



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BoTest™ BoNT Detection Reporters: *in vitro* detection of serotypes A, B, D, E, F, and G

Measurements:

- Responds to BoNT proteolytic activity
- Two emission wavelengths: 470 and 527 nm
- Ratiometric assay reduces well-to-well variation, errors associated with pipetting
- Equipment: Plate reader (two wavelength capabilities)

Mix-and-read, no secondary reagents

BoTest™ A/E and BoTest™ B/D/F/G BoNT Detection Kits

- Released in 2009 and 2010
- Used by government and commercial labs
- Validated by pharmaceutical client for quantification of BoNT/A drug product
- Used by USAMRIID and USAMRICD for the discovery and characterization of BoNT inhibitors
- Kit contains: BoTest™ reporter, 10x Reaction buffer, protocol, CofA
- BioSentinel publication: Ruge et al (2011) *Analytical Biochemistry*, 411: 200

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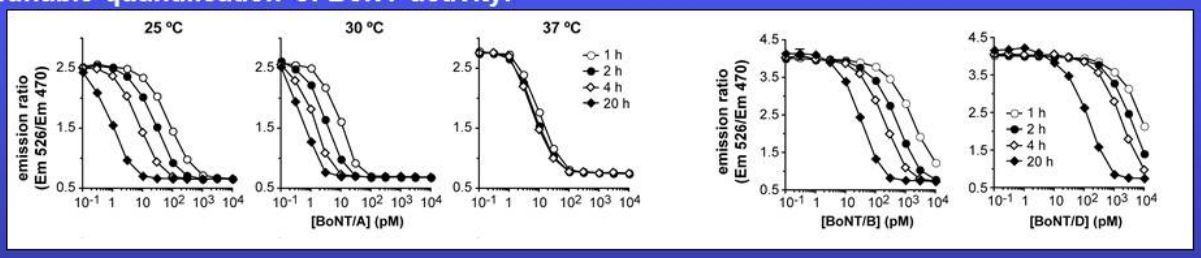
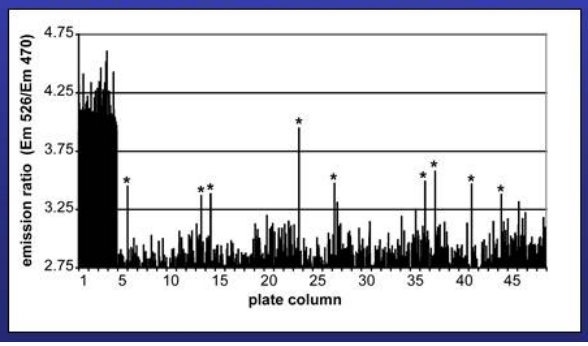
BoTest™ BoNT Detection Reporters: *in vitro* detection of serotypes A, B, D, E, F, and G

Tunable quantification of BoNT activity:



Discovery and characterization of BoNT inhibitors:



150,000+ compounds screened to date

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Limits of detection for the BoTest™ BoNT Detection Assays.

Table 1. Limits of detection for the BoTest™ reporters. (Values in pM BoNT)

		BoTest™ A/E			BoTest™ B/D/F/G				
temp.	time (h)	BoNT/A lot 1	BoNT/A lot 2	BoNT/E_{tryp}	BoNT/B	BoNT/B_{tryp}	BoNT/D	BoNT/F	BoNT/G_{tryp}
25 °C	1	10	30	3	1000	100	1000	100	>10000
	2	3	10	3	100	100	1000	100	10000
	4	3	3	1	100	30	300	30	1000
	20	0.3	0.3	1	10	3	100	3	30
30 °C	1	3	10	3	100	100	1000	100	>10000
	2	1	10	3	30	10	300	100	300
	4	0.3	3	1	30	10	300	30	300
	20	0.3	0.3	0.3	10	3	100	3	30
37 °C	1	3	10	3	100	30	1000	100	>10000
	2	3	10	3	30	10	300	30	300
	4	3	10	1	30	10	300	30	300
	20	ND	3	1	10	3	30	10	30

In 100 µl, 300 fM = 4.6 pg = ~1.3 mLD_{50} for BoNT/A

In 100 µl, 3 pM = 45 pg = ~4.5 mLD_{50} for BoNT/B

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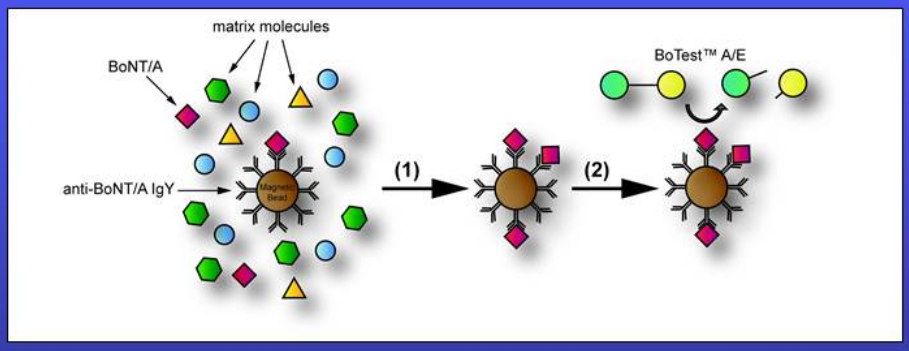
BoTest™ Matrix: Detection and quantification of BoNT in complex matrices



BoTest™ Matrix A and E BoNT Detection Kits

- **BoTest™ Matrix A released in May 2011; BoTest™ Matrix E to be release in summer 2011, currently available for pre-release testing.**
- **POC (brief) completed with pharmaceutical client**
- **Demonstrated use with field samples**
- **Kit contains: BoTest™ reporter, Matrix Beads, 10x Matrix Binding Buffer, 10x Matrix Wash Buffer, 10x Reaction buffer, protocol, CofA**

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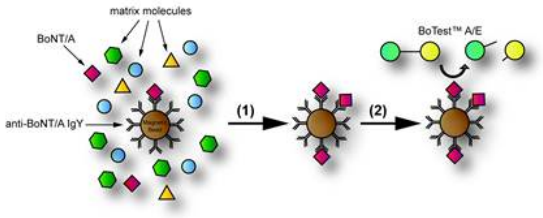
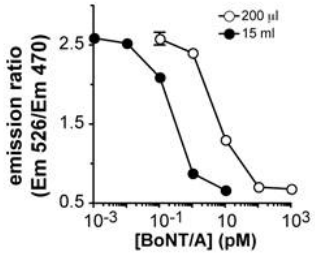
Detection of BoNT in large or small volumes





BoNT effectively concentrated by Matrix

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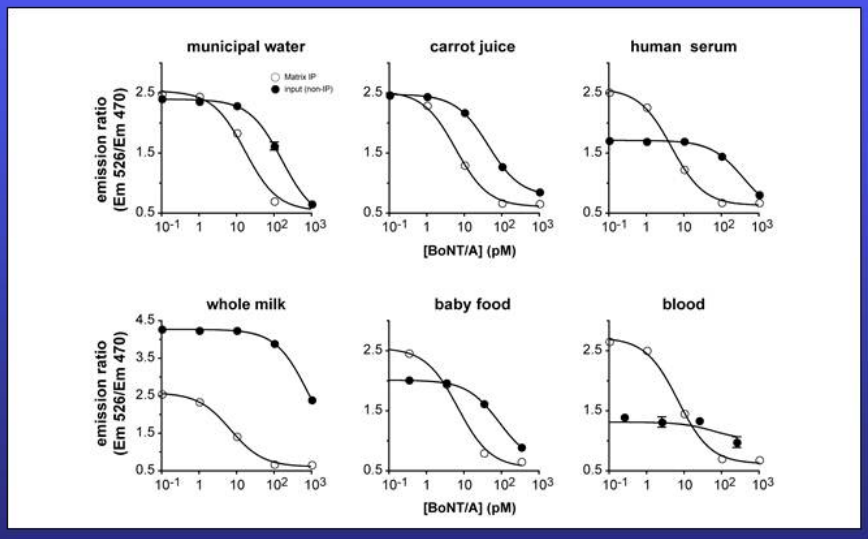
Detection of BoNT/A in milk, carrot juice, whole blood, tap water, baby food, and human serum



Femtomolar to picomolar sensitivity in complex matrices

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Detection of BoNT/A in milk, carrot juice, whole blood, tap water, and human serum

Limit of Detection	2 Hours	4 Hours	24 Hours
Water	< 100 fM	< 100 fM	< 100 fM
Carrot Juice	< 1 pM	< 1 pM	< 100 fM
Baby Food	< 3.33 pM	< 333 fM	< 333 fM
100% Human Serum	< 1 pM	< 1 pM	<100 fM
Whole Milk	< 10 pM	< 1 pM	< 1 pM
Control	< 100 fM	< 1 pM	< 100 fM

Femtomolar to picomolar sensitivity in complex matrices

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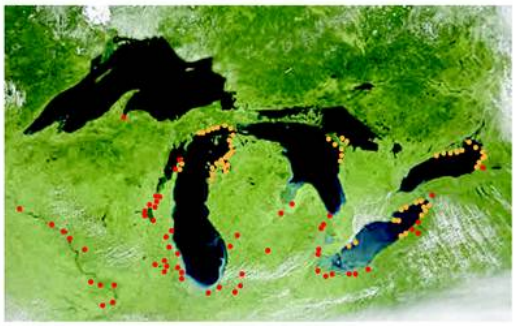
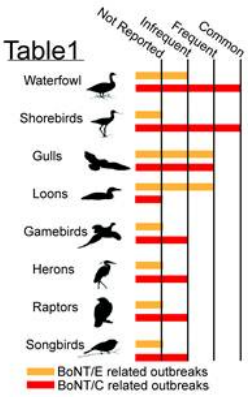
Detection of BoNT/E in avian samples

Figure 1. Avian Botulism

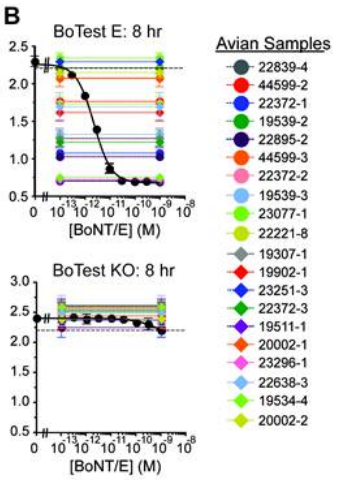
A



Modified from : Friend, M. (2006) Disease Emergence and Resurgence: The Wildlife-Human Connection: Reston, Va, US Geological Survey, Circular, 1285:26. and USGS Data (2007).



B



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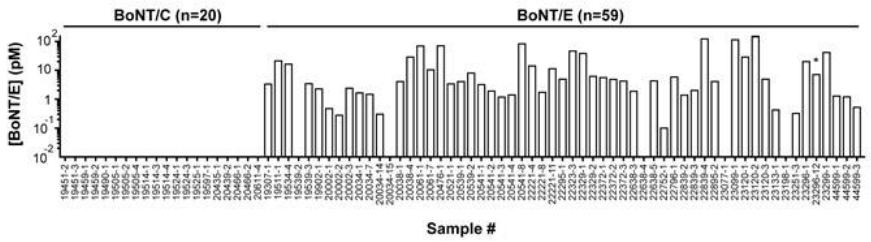
Detection of BoNT/E in avian samples



Complete Sample Set (n=79)

Method	BoNT/E Positive	BoNT/E Negative	False Negative	False Positive	*Equivocal	Diagnostic Specificity (%)	Diagnostic Sensitivity (%)
BoTest E	53	25	5	0	1	100	91.4
Mouse Assay	59	20	--	--	--	--	--

The BoTest™ Matrix assay is compatible with field samples.

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BoCell™: A fluorometric cell-based assay for detection of BoNTs

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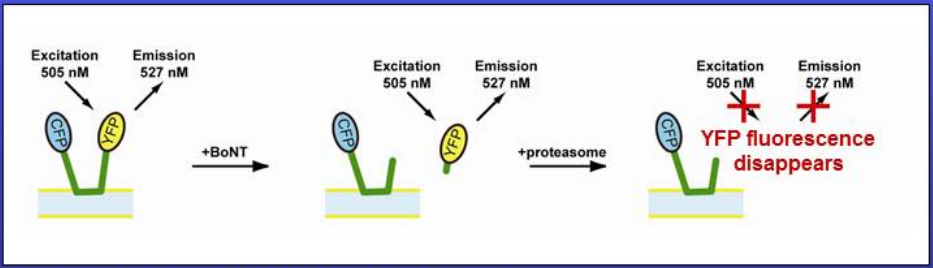
Cell-based assay advantages

- Simulates pathogenesis of natural disease at a cellular level
- Potential replacement for mouse bioassay
- Discover and test BoNT inhibitors at a cellular level
- Understand BoNT cell biology



Dong et al (2004) *PNAS* **101:** 14701

BoCell™ A Cell-based assay

- Official release in summer 2011, available now for pre-release testing and licensing
- Engineered and highly selected cell line
- Will be offered as a licensed product only
- POC with drug product to start in May 2011 (pharma supported)

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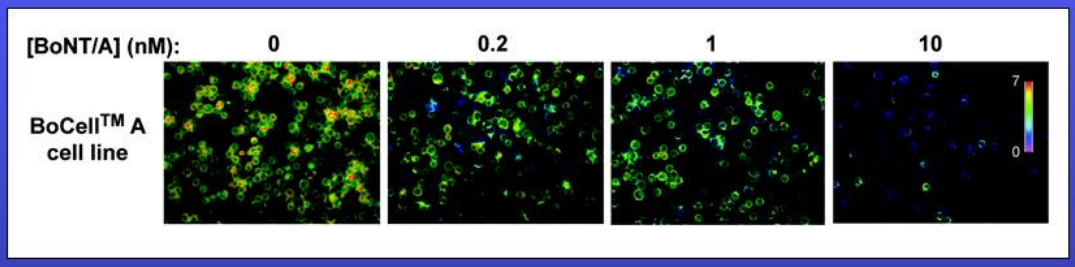
Fluorescence emissions of BoCell™



Measurement

- Responds to BoNT proteolytic activity
- Two emission wavelengths: 470 and 527 nm (no FRET)
- Ratiometric assay reduces well-to-well variation, errors associated with uneven cell density
- Equipment: Plate reader (two wavelength capabilities), microscope, or HC imager

BoCell™ designed to be used with minimal equipment and training.

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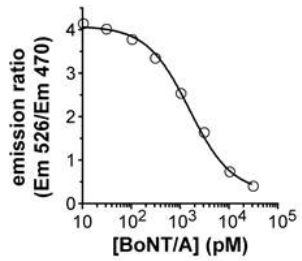
Statistical performance of the BoCell™ Assay in a microplate reader

A



LOD = 39.5 pM (219 total mLD50 units)
LOQ = 133 pM (740 total mLD50 units)

B

[BoNT/A] (pM)	average emission ratio	SD	% CV
0	4.15	0.05	1.3
10	4.15	0.12	2.8
30	4.03	0.09	2.2
100	3.79	0.06	1.6
300	3.36	0.05	1.4
1000	2.55	0.06	2.4
3000	1.65	0.05	3.0
10000	0.74	0.08	10.4
30000	0.41	0.06	13.7

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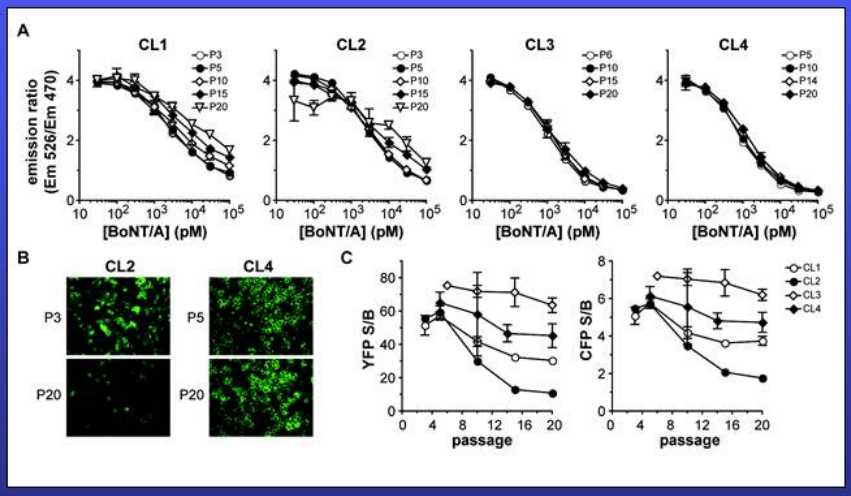
Performance and stability of the engineered BoCell™ cell lines



BoCell™ engineered for stability and reproducibility.

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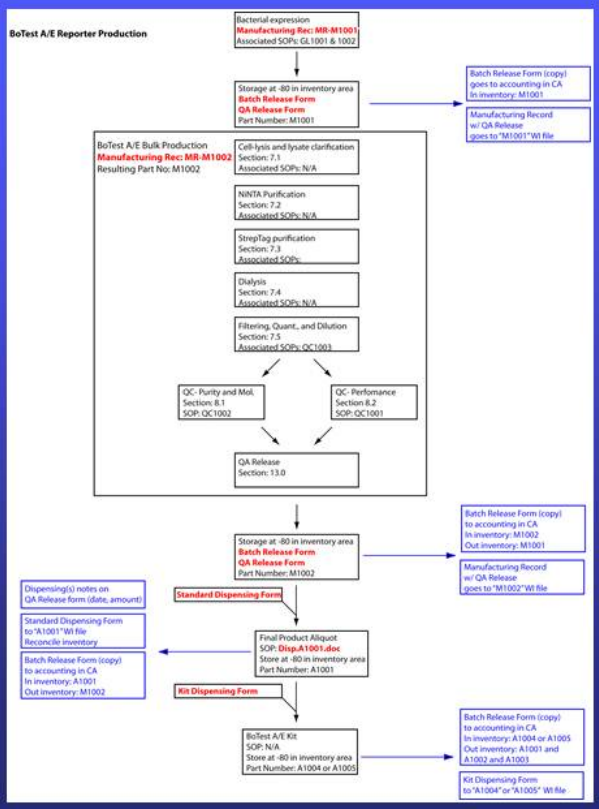
Manufacturing, Quality Assurance, and Product Validation

Goals:

- Complete product and raw materials tracking
- Consistent product
- Tailored to client needs
- Guaranteed results



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Manufacturing, Quality Assurance, and Product Validation

Manufacturing and Quality Assurance:

- Manufacturing Records: step-by-step, materials used, calculations made, etc
- Quality Control: embedded in manufacturing records, detailed in separate SOPs
- SOPs: centrally controlled
- Parts Number and Lot system: complete materials tracking
- QA Documentation: specification review
- Certificates of Analysis
- Equipment maintenance: SOPs, critical certifications, and documentation
- Quality Assurance Policy and Documentation

Methods and Protocol Qualification (Validation):

- Accelerated Short-term Stability
- Long-term Stability (2 year)
- Freeze-thaw cycling
- Robustness testing
- Effects of Solvents
- Effects of Different BoNT Manufacturers and Forms

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Research & Management Talent

- Ward Tucker, PhD, Research Director, BioSentinel
- Dhammika Atapattu, MD, PhD, Scientist and Group Leader, BioSentinel
- Daniel Ruge, MS, Scientist and Lab Manager, BioSentinel
- Mark Dunning, PhD, Scientist and Group Leader, BioSentinel
- Heidi Olivares, BS, Scientist, BioSentinel
- Timothy Piazza, PhD, Post-doctoral Fellow, BioSentinel/UW/NWHC
- Michael Cannon, Managing Partner BioSentinel
- Benjamin Lap, Managing Partner BioSentinel
- Füsûn Naomi Zeytin, PhD, Founder and CSO BioSentinel

- Dr. Bill Checovich, Product and Business Development Consultant, Madison, WI
- Ed Chapman, Ph.D. Investigator, Howard Hughes Medical Institute Professor, Department of Physiology, UW and Chief Scientific Consultant, BioSentinel
- Dr. David Blehert, National Wildlife Health Center, US Geological Survey
- Jack A. Heinemann, Director Wisconsin Security Research Consortium (WSRC)
- Richard Kruger, PhD, Regulatory Affairs Consultant, Kruger Consulting Inc.

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Collaborators

- USGS-National Wildlife Health Center (Madison)
- University of Wisconsin-Madison
- USAMRIID
- USAMRICD
- Genetel (Madison)
- Life Technologies (Madison)
- Tufts University
- Synaptic Research
- Metabiologics (Madison)
- Battelle Memorial Institute
- USDA-ARS
- AFIP

Madison, WI has the highest concentration of botulinum-related research laboratories and businesses than anywhere else in the world.